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Mediation of Corticotropin Releasing Factor Type 1 Receptor Phosphorylation and Desensitization by Protein Kinase C: A Possible Role in Stress Adaptation

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ABSTRACT

Protein kinase C (PKC)-mediated desensitization of the corticotropin releasing factor type 1 (CRF_1) receptor was investigated in human retinoblastoma Y79 and transfected COS-7 cells. Because stimulation of Y79 cells with CRF resulted in large (~30-fold) increases in intracellular cAMP accumulation without changing inositol phosphate levels, the CRF_1 receptor expressed in retinoblastoma cells couples to $G_{s,}$ but not to G_q, and predominantly signals via the protein kinase A cascade. Direct activation of PKC by treatment with the phorbol ester phorbol 12-myristate 13-acetate (PMA) or 1,2-dioctanoyl-*sn*-glycerol (DOG) desensitized CRF_1 receptors in Y79 cells, reducing the maximum for CRF- (but not forskolin)-stimulated cAMP accumulation by 56.3 ± 1.2% and 40.4 ± 2.1%, respectively ($p < 0.001$). Pretreating Y79 cells with the PKC inhibitor bisindolylmaleimide I (BIM) markedly inhibited PMA's desensitizing action on CRF-stimulated cAMP accumulation, but did not affect homologous CRF_1 receptor desensitization. Retinoblastoma cells were found to express PKCα, βI, βII, δ, λ, and RACK1. When α and β isoforms of PKC were down-regulated 80 to 90% by a 48-h PMA exposure, PMA-induced CRF_1 receptor desensitization was abolished. In transfected COS-7 cells the magnitude of CRF_1 receptor phosphorylation after a 5-min exposure to PMA was 2.32 ± 0.21-fold greater compared with the basal level. Pretreating COS-7 cells with BIM abolished PMA-induced CRF_1 receptor phosphorylation. These studies demonstrate that protein kinase C (possibly α and β isoforms) has an important role in the phosphorylation and heterologous desensitization of the CRF_1 receptor.

Cellular signaling mediated by agonist-induced G protein-coupled receptor (GPCR) activation must be stringently regulated to prevent an unrestrained level of receptor stimulation. Two major modes of terminating GPCR signaling have been identified as homologous and heterologous desensitization. Homologous desensitization refers to an agonist-dependent mechanism of cellular adaptation whereby signal transduction becomes rapidly terminated in the continued presence of high agonist concentrations. This begins with the selective translocation of a G protein-coupled receptor kinase (GRK) from the cytosol to the membrane, where it phosphorylates specific serine and threonine residues in the GPCR's C-terminal tail and/or third intracellular loop (Penn and Benovic, 1998; Cliang et al., 2002; Pierce et al., 2002; Kohout and Lefkowitz, 2003).

The neuropeptide CRF regulates behavioral, neuroendocrine, and autonomic responses to stress by acting at two high-affinity CRF receptors (CRF_1 and CRF_2) in the amygdala and its extended neurocircuits, as well as in adrenocorticotropin-secreting pituitary corticotropes (Grigoriadis et al., 2001; Dautzenberg and Hauger, 2002; Perrin and Vale, 2002). CRF_1 receptors are rapidly desensitized in retinoblas-

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ABBREVIATIONS: GPCR, G protein-coupled receptor; GRK, GPCR kinase; CRF, corticotropin releasing factor; CRF_1, CRF receptor type 1; PKA, protein kinase A; G_s, stimulatory GTP binding protein; PKC, protein kinase C; BSA, bovine serum albumin; PMA, phorbol 12-myristate 13-acetate; DOG, 1,2-dioctanoyl-*sn*-glycerol; BIM, bisindolylmaleimide I; DMEM, Dulbecco's modified Eagle's medium; IP, inositol phosphate; ANOVA, analysis of variance; RACK, receptors for activated C-kinase; HA, hemagglutinin; PAC_1, pituitary adenylate cyclase-activating polypeptide type 1 receptor.

toma Y79, neuroblastoma IMR-32, and transfected fibroblast Ltk cells exposed to high agonist concentrations of CRF or urocortin1 (Dieterich et al., 1996; Hauger et al., 1997; Dautzenberg et al., 2001a, 2002; Roseboom and Kalin, 2001). Our data indicate that GRK3 plays an important role in the homologous desensitization of CRF_1 receptors. First, in Y79 cells, uptake of a GRK3 antisense oligonucleotide or transfection of a GRK3 antisense cDNA construct decreased GRK3 expression by ~55% and inhibited homologous CRF_1 receptor desensitization by ~65% (Dautzenberg et al., 2001a). In addition, a large increase in GRK3 expression occurs in Y79 cells during the emergence of CRF-induced CRF_1 receptor desensitization (Dautzenberg et al., 2002). We have also observed that epitope-tagged CRF_1 receptors expressed in COS-7 cells are rapidly phosphorylated following exposure to a saturating concentration of CRF (Hauger et al., 2000). Since COS-7 cells express GRK2, but not GRK3 (Menard et al., 1997), it appears that GRK2 also has a role in desensitizing CRF_1 receptors. These findings suggest that GRK3, and possibly GRK2, regulate CRF_1 receptor signaling when the release of endogenous agonists in the central nervous system is increased by stress.

In contrast to homologous desensitization, heterologous mechanisms can attenuate receptor responsiveness independent of agonist binding. Typically, heterologous desensitization occurs when an unactivated receptor is phosphorylated via the catalyzing action of a specific second messenger kinase that has been stimulated by another GPCR (Penn and Benovic, 1998; Pierce et al., 2002). Several studies have shown that protein kinase A (PKA) desensitizes many G_s-coupled GPCRs by phosphorylating intracellular consensus sites (Krupnick and Benovic, 1998; Penn and Benovic, 1998; Pierce et al., 2002; Kohout and Lefkowitz, 2003). Because the agonist-induced "active" conformation of the CRF_1 receptor couples to G_s, which in turn activates the cAMP-PKA pathway, it was initially predicted that PKA would have a predominant role in CRF_1 receptor desensitization (Chen et al., 1993). However, neither maximal PKA stimulation nor PKA inhibition influenced CRF_1 receptor phosphorylation and desensitization (Hauger et al., 2000; Dautzenberg et al., 2001a; Roseboom and Kalin, 2001). Thus, PKA does not appear to mediate homologous or heterologous desensitization of the CRF_1 receptor.

Protein kinase C (PKC) can also mediate homologous or heterologous GPCR desensitization. PKC-mediated phosphorylation of GPCR and adenylyl cyclase proteins contributes to desensitization, internalization, and down-regulation of many receptors (Penn and Benovic, 1998; Oppermann et al., 1999; Olivares-Reyes et al., 2001; Bhattacharyya et al., 2002; Mandyam et al., 2002; Pierce et al., 2002). PKC-mediated mechanisms have important roles in regulating ion channel sensitivity and other aspects of postsynaptic neurotransmission, including long-term potentiation and depression (Tanaka and Nishizuka, 1994). PKC-related signaling abnormalities have also been implicated in the pathophysiology of a wide variety of human illnesses (Tanaka and Nishizuka, 1994; Dempsey et al., 2002).

Two potential PKC phosphorylation sites (Ser^{386} and Ser^{408}) are present in the cytoplasmic C-terminal tail of the CRF_1 receptor (Chen et al., 1993; Dautzenberg et al., 2001b). A recent study has shown that oxytocin receptor activation initiates heterologous desensitization of CRF-stimulated cAMP accumulation in human myometrial cells via a PKC-dependent mechanism (Grammatopoulos and Hillhouse, 1999). Because CRF_1 receptors have been shown to couple to G_q proteins and signal via the PKC cascade (Ulisse et al., 1990; Kiang et al., 1994; Dieterich et al., 1996; Karteris et al., 2000), PKC may also contribute to homologous CRF_1 receptor phosphorylation and desensitization. The primary goal of this study was to test the hypothesis that protein kinase C mediates phosphorylation and desensitization of the CRF_1 receptor.

Materials and Methods

Materials. Reagent purchases were as follows. 1) Bovine serum albumin (BSA, fraction V), isobutylmethylxanthine, forskolin, and other highly pure chemicals: Sigma-Aldrich (St. Louis, MO); 2) aprotinin (Trasylol), phorbol 12-myristate 13-acetate (PMA), 4α-phorbol 12-myristate 13-acetate (an inactive PMA isomer), 1,2-dioctanoyl-*sn*-glycerol (DOG), bisindolylmaleimide I (BIM), staurosporine: Calbiochem (San Diego, CA); 3) defined fetal bovine serum (SH30070.03): Hyclone Laboratories (Logan, UT). The University of California, San Diego Cell Culture Core Facility supplied all other cell culture reagents (Mediatech, Herndon, VA). Ovine CRF (CRF: Bachem California, Torrance, CA; purity >98%) was used to stimulate cAMP accumulation in all experiments and to desensitize CRF_1 receptors. All SDS-PAGE reagents were purchased from Invitrogen-Novex (Carlsbad, CA). For CRF_1 receptor phosphorylation experiments, the following reagents were used: 1) protein A-Sepharose (PrA-Seph) (Oncogene Research Products, San Diego, CA); 2) HA.11 mouse monoclonal anti-HA antibody (Babco, Richmond, CA).

Cell Culture and Transfection. Suspension human retinoblastoma Y79 cultures were grown at a density of 5 to 8 $\times$ 10^7 cells/flask in RPMI 1640 and used between passages 4 and 25 as previously described (Hauger et al., 1997; Dautzenberg et al., 2001a). COS-7 cells were seeded at 6 $\times$ 10^5 cells/10-cm dish in DMEM containing 10% (v/v) fetal bovine serum, 100 μg/ml streptomycin, and 100 IU/ml penicillin (COS-7 cell medium). COS-7 cells were cultured for 3 days before transfection using 5 ml of OptiMEM containing 10 μg/ml LipofectAMINE (Invitrogen, Carlsbad, CA) and 5 μg of HA-tagged CRF_1 receptor cDNA for 6 h at 37°C as described (Hauger et al., 2000). After changing to fresh COS-7 cell medium, the cells were cultured for a further 2 days before use.

Second Messenger Assays. Following extensive cell washing, intracellular cAMP levels were measured in ether-extracted and acetylated cell lysates using a double-antibody radioimmunoassay kit (cAMP [^{125}I] assay system, RPA 509; Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK), as previously described (Hauger et al., 1997; Dautzenberg et al., 2001a). For inositol phosphate (IP) experiments, Y79 cells were metabolically labeled with myo-[2-^{3}H]inositol (5 μCi/ml) overnight (18 h). After labeled cells were washed twice with a large volume (40 ml) of myoinositol-free RPMI 1640, they were preincubated in myoinositol-free Medium 199(E) with 10 mM lithium chloride for 30 min at 37°C. After cells were again washed, centrifuged, and resuspended in myoinositol-free Medium 199(E) with 10 mM lithium chloride, they were maximally stimulated with 1 μM CRF for 20 min. After the reaction was stopped by adding 10 mM formic acid, formation of IP_2 and IP_3 was measured by anion exchange chromatography using Bio-Rad AG 1-X8 columns, as previously described (Olivares-Reyes et al., 2001).

Western Blot Quantitation of PKC Protein Expression. Lysates of Y79 cells (20–30 μg per lane) were loaded onto 4 to 12% Tris-glycine gradient gels (Invitrogen-Novex) and proteins were resolved in a NOVEX Xcell II Mini-Cell System using SDS-PAGE under reducing conditions (Invitrogen-Novex Tris-glycine SDS sample buffer containing 5.0% β-mercaptoethanol) at a fixed 125 V (current 35–40 amps) for 90 min according to the method of Laemmli (Dautzenberg et al., 2001a; Dautzenberg and Hauger, 2001). After

Western transfer of resolved retinoblastoma proteins onto polyvinylidine difluoride membranes (Invitrogen-Novex) was completed (Dautzenberg et al., 2001a; Dautzenberg and Hauger, 2001), blots were blocked for 1 h in a solution of Tris-buffered saline (20 mM Tris pH 7.5, 500 mM NaCl) with 0.2% Tween 20 and 4% BSA (TTBS-BSA) with constant shaking at room temperature. Blots were then washed with TTBS-BSA and immunoprobed overnight (~18 h) at 4°C with constant shaking with one of the following antibodies: 1) a mouse monoclonal antibody targeting PKCα (610108; 1:1,000), PKCγ (611158; 1:1,000), PKCδ (610397; 1:500), PKCϵ (610085; 1:1,000), PKCη (610814; 1:250), PKCθ (610089; 1:250), PKCι (610175; 1:250), PKCλ (610207; 1:250), RACK1 (610178; 1:2,500), or DGKθ (610930; 1:250) (BD Biosciences PharMingen Transduction Laboratory; San Diego, CA); 2) a mouse monoclonal targeting PKCβI (E-3/sc-8049; 1:100) or a rabbit polyclonal targeting PKCβII (C-18/sc-210; 1:200) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); or 3) a rabbit polyclonal antibody (P500; 1:3,000, kindly provided by Dr. A. Newton, Department of Pharmacology, University of California, San Diego) targeting the phosphorylated activation loop of protein kinase CβII (Dutil and Newton, 2000). After the membranes were washed in TTBS-BSA (six 10-min washes), blots were incubated for 1 h at room temperature with constant shaking with one of the following antibodies (in TTBS-BSA): 1) sheep anti-mouse IgG-HRP (NA931, 1:5,000; Amersham Biosciences Inc., Piscataway, NJ) or 2) donkey anti-rabbit IgG-HRP (NA934, 1:5,000; Amersham Biosciences Inc.). After membranes were washed extensively in TTBS (six 20-min washes), chemiluminescent detection of Western blots was performed using ECL$^+$ Plus (Amersham Biosciences Inc.).

CRF_1 Receptor Phosphorylation Assay. Phosphorylation of the CRF_1 receptor was determined as previously described (Hauger et al., 2000). Briefly, transfected COS-7 cells in 10-cm dishes were metabolically labeled for 4 h at 37°C in 5 ml of P_i-free DMEM containing 0.1% (w/v) BSA and 100 μCi/ml $^{32}P_i$. Cells were then pretreated with vehicle or BIM for 30 min followed by a 5-min exposure to PMA for 5 min. Treated cells were lysed in buffer (LB: 50 mM Tris, pH 8.0, 100 mM NaCl, 20 mM NaF, 10 mM sodium pyrophosphate, 5 mM EDTA, 10 mg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml pepstatin, 10 μg/ml benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 μM okadaic acid) and probe-sonicated twice for 20 s. After removal of nuclei at 750*g*, membranes were pre-extracted by the addition of an equal volume of LB containing 2 M NaCl and 8 M urea followed by overnight tumbling at 4°C. The membranes were then collected at 200,000*g* and solubilized in LB+ [LB supplemented with 1% (v/v) NP 40, 1% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS] with Dounce homogenization. After clarification at 14,000*g*, solubilized membranes were precleared by being incubated with 2% (v/v) protein A-Sepharose for 1 h at 4°C. Immunoprecipitation of CRF_1 receptors was performed by adding 1 μl of HA.11 antibody and 2% (v/v) protein A-Sepharose and incubating overnight at 4°C. After washing of the Sepharose-bound immune complexes in LB+ lacking protease inhibitors, ^{32}P-labeled phospho-HA-CRF_1 receptors were eluted in Laemmli sample buffer for 1 h at 48°C and resolved by SDS-PAGE (8–16% gradient resolving gel) before visualization in a PhosphorImager (Amersham Biosciences Inc.).

Data Reduction and Statistical Analyses. CRF_1 receptor desensitization data were calculated as percentage of control values as previously described (Hauger et al., 1997). Data reduction for the cyclic AMP radioimmunoassay was performed using a log-logit program. Analyses of variance (ANOVAs) across experimental groups were performed on a MacIntosh PC using PRISM Version 2.0 (GraphPad Software Inc., San Diego, CA). If the one-way ANOVA was statistically significant, planned post hoc analyses were performed using Bonferroni's multiple comparison tests to determine individual group differences. Immunoreactive PKC protein bands on Western blots were quantitated and analyzed on the STORM imager using ImageQuant software (Amersham Biosciences Inc.) (Dautzenberg et al., 2001a).

Results

Time Course for PMA-Induced CRF_1 Receptor Desensitization in Y79 Cells. We began our study by investigating potential PKC regulation of CRF_1 receptor signaling in human retinoblastoma Y79 cells. Although PMA-induced PKC activation augments the ability of CRF to stimulate cAMP accumulation and adrenocorticotropin release in anterior pituitary cells (Abou-Samra et al., 1987), the magnitude of cAMP accumulation stimulated by 100 nM CRF was decreased by ~50% when Y79 cells were coincubated with 100 nM PMA during the 15-min stimulation period (Fig. 1A). When Y79 cells were stimulated with 1 μM CRF for 20 min, inositol phosphate levels were not increased above basal (i.e., no CRF stimulation) values (Fig. 1B) in contrast to a 29.5 ± 0.9-fold increase in cAMP formation observed in CRF-stimulated cells ($p < 0.0001$; data not shown). Previous studies have shown that PKC participates in both homologous and heterologous GPCR desensitization by promoting the phosphorylation of serines and threonines within consensus sites located in a receptor's C terminus and/or other intracellular loops (Penn and Benovic, 1998; Pierce et al., 2002). When Y79 cells were pretreated for 1 h with 1 μM PMA to maximally activate PKC, the magnitude of cAMP accumulation following subsequent restimulation with 100 nM CRF was decreased by ~50% ($p < 0.0001$) (Fig. 1C), thereby providing evidence for PKC mediation of CRF_1 receptor desensitization. When the reversibility of PMA-induced desensitization of CRF_1 receptors was investigated, we found that CRF-stimulated cAMP stimulation failed to recover from the desensitized state in Y79 cells that were exposed to 1 μM PMA for 1 h, washed extensively, and then cultured for an additional 4 h (data not shown). The specificity of PMA-induced CRF_1 receptor desensitization was confirmed by demonstrating that CRF-stimulated cAMP accumulation was not significantly reduced following a 1-h pretreatment with 1 μM 4α-phorbol, a PMA analog that is incapable of activating PKC (Fig. 1C). Although Y79 cells were washed thoroughly at the end of the pretreatment period, basal cAMP levels were significantly increased ($p < 0.001$) following a 1-h exposure to PMA but not 4α-phorbol (Fig. 1).

When the time course of the desensitizing effect of PMA pretreatment was analyzed, CRF-stimulated cAMP accumulation was found to decrease progressively in Y79 cells exposed to 1 μM PMA for 5 min (22.7 ± 1.6%), 10 min (48.9 ± 2.2%), 15 min (48.8 ± 2.6%), and 30 min (58.2 ± 2.6%) (Fig. 2A). The magnitude of PMA-induced CRF_1 receptor desensitization was similar in Y79 cells exposed to PMA for 30 min or 1 h (56.3 ± 1.2%) (Fig. 2A). However, the reduction in CRF-stimulated cAMP accumulation was significantly less following exposure to PMA for 3 h (42.2 ± 1.2%; $p < 0.01$) compared with 1 h (Fig. 2A). Although PMA pretreatment markedly reduced cAMP accumulation in Y79 cells rechallenged with CRF, it did not significantly decrease forskolin-stimulated cAMP accumulation during the same time period (Fig. 2A). When full CRF concentration-response curves were generated, the maximum CRF-stimulated cAMP accumulation was decreased ~50% in Y79 cells exposed to 1 μM PMA (14.0 pmol/106 cells) compared with control cells (28.8 pmol/106 cells) (Fig. 2B).

The time course for homologous CRF_1 receptor desensitization in Y79 cells exposed to 1 μM CRF for 5 min to 3 h

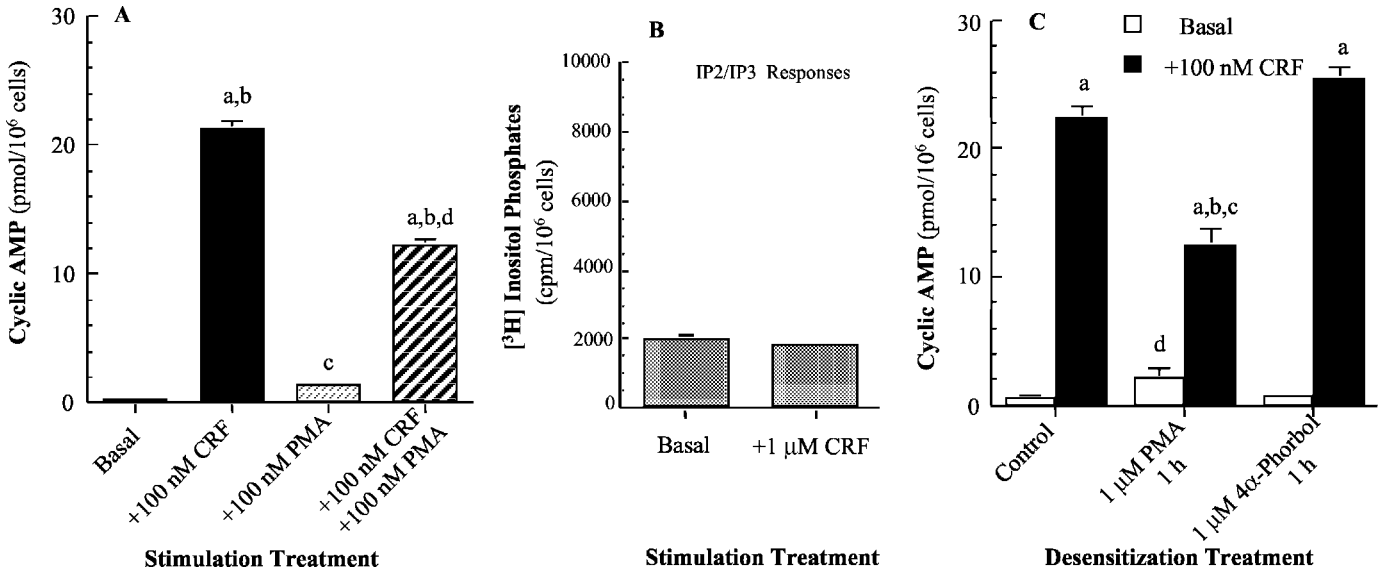

Fig. 1. Effect of PMA exposure on CRF-stimulated cAMP accumulation in Y79 cells. A, effect of coincubation with 100 nM CRF and/or 100 nM PMA during the 15-min stimulation period. Each bar represents mean ± S.E.M. values of cAMP levels ($n = 12$ per group). By ANOVA, there were significant differences across the groups for changes in cAMP levels ($F = 747.4$, $p < 0.0001$). The following post hoc differences were found to be statistically significant between cell groups: a, $p < 0.001$ versus basal; b, $p < 0.001$ versus 100 nM PMA; c, $p < 0.05$ versus basal; d, $p < 0.001$ versus 100 nM CRF. B, effect of incubation with 1 μM CRF on inositol phosphate generation during a 20-min stimulation period. No significant differences ($F = 2.52$; $p = 0.10$) in IP_2/IP_3 levels were observed in CRF-stimulated or basal cells. C, effect of pretreatment with 1 μM PMA or 1 μM 4α-phorbol for 1 h before a 15-min stimulation with 100 nM CRF. Each bar represents mean ± S.E.M. values of cAMP levels ($n = 5$ per group). Retinoblastoma CRF_1 receptors were desensitized by 46% during the 1-h exposure to PMA, but not 4α-phorbol. By ANOVA, there were significant differences across the groups for changes in cAMP levels ($F = 117.7$, $p < 0.0001$). The following post hoc differences were found to be statistically significant between cell groups: a, $p < 0.001$ versus basal; b, $p < 0.001$ versus control-CRF; c, $p < 0.001$ versus 4α-phorbol-CRF; d, $p < 0.001$ versus control-basal or 4α-phorbol-basal.

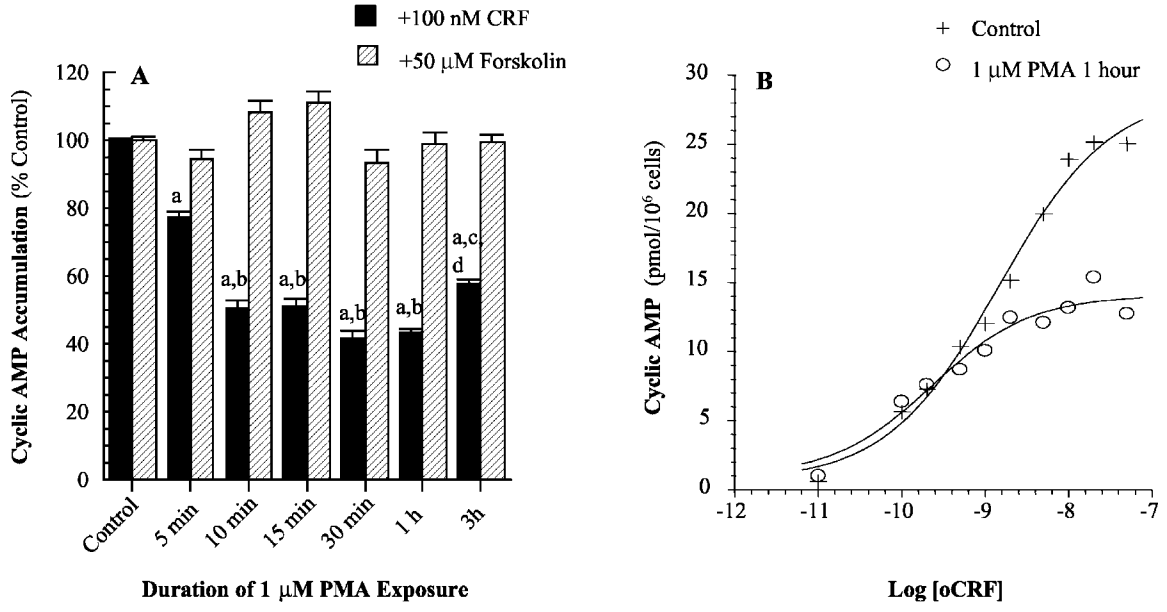

Fig. 2. Time course for CRF_1 receptor desensitization in Y79 cells exposed to 1 μM PMA. After pretreatment with PMA for the 5 min to 3 h was completed, retinoblastoma cells were extensively washed and stimulated with 100 nM CRF for 15 min. A, PMA-induced desensitization: data are mean ± S.E.M. of values expressed as percentage of control collected in 19 separate experiments ($n = 3$–12 replicates per treatment group). In control cells (i.e., no PMA pretreatment), basal and CRF-stimulated cAMP levels were 0.68 ± 0.13 and 22.57 ± 0.79 pmol/10^6 cells, respectively. By ANOVA, there were significant differences across the groups ($F = 182.4$, $p < 0.0001$). The following post hoc differences were found to be statistically significant between groups: a, $p < 0.001$ versus control; b, $p < 0.001$ versus 5-min PMA; c, $p < 0.05$ versus 5-min PMA; d, $p < 0.01$ versus 1-h PMA. B, effect of pretreating Y79 cells with 1 μM PMA for 1 h on the sensitivity (EC_{50}) and maximum for the dose-response stimulation of intracellular cAMP accumulation by CRF. In this representative experiment, cAMP levels (picomoles per 10^6 cells) were measured in duplicate in PMA-pretreated and control cells stimulated with 0 to 100 nM CRF.

revealed progressive reductions in CRF-stimulated cAMP accumulation ($p < 0.0001$) (Fig. 3). However, in contrast to the time course of PMA-induced CRF_1 receptor desensitization (Fig. 2A), the reduction in CRF-stimulated cAMP accumulation was significantly greater in Y79 cells exposed to 1 μM CRF for 3 h (65.4 ± 1.8%; $p < 0.05$) compared with 1 h (49.3 ± 2.8%) (Fig. 3). The stimulation of cAMP accumulation by 50 μM forskolin in Y79 cells desensitized by CRF did not differ from cAMP responses to forskolin in control cells (data not shown), as previously observed (Hauger et al., 1997).

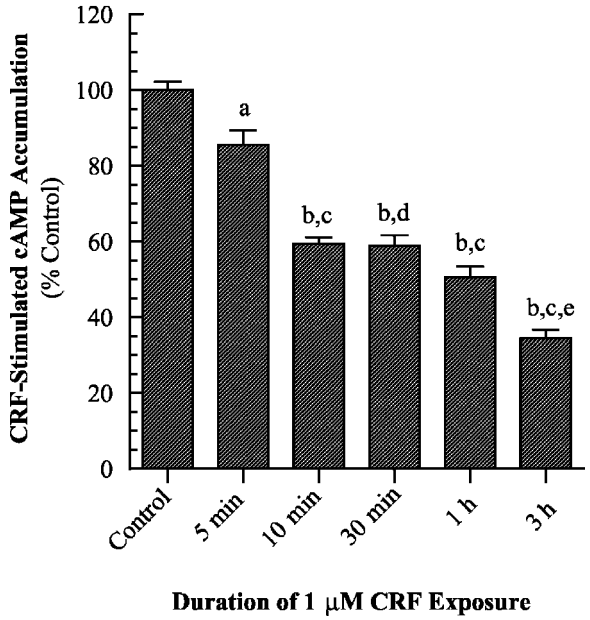

Fig. 3. Homologous CRF_1 receptor desensitization induced by 1 μM CRF: data are mean ± S.E.M. of values expressed as percentage of control collected in seven separate experiments (n = 4–13 replicates per treatment group). In control cells (i.e., no CRF pretreatment), basal and CRF-stimulated cAMP levels were 0.63 ± 0.06 and 22.41 ± 0.75 pmol/10^6 cells, respectively. By ANOVA, there were significant differences across the groups (F = 102.3, $p < 0.0001$). The following post hoc differences were found to be statistically significant between groups: a, $p < 0.05$ versus control; b, $p < 0.001$ versus control; c, $p < 0.001$ versus 5-min CRF; d, $p < 0.01$ versus 5-min CRF; e, $p < 0.05$ versus 1-h CRF.

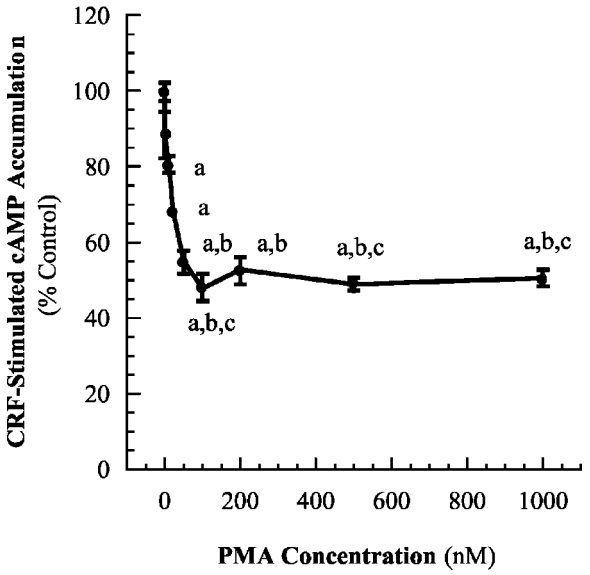

Fig. 4. Concentration-dependence of PMA-induced CRF_1 receptor desensitization in Y79 cells for a 15-min pretreatment period. Data are mean ± S.E.M. of values expressed as percentage of control replicated in two independent experiments (n = 4–18 replicates per treatment group). In control cells (i.e., no PMA pretreatment), basal and CRF-stimulated cAMP levels were 0.77 ± 0.07 and 20.85 ± 0.36 pmol/10^6 cells, respectively. By ANOVA, there were significant differences across the groups (F = 52.6, $p < 0.0001$). The following post hoc differences were found to be statistically significant between groups: a, $p < 0.001$ versus control; b, $p < 0.001$ versus 10 nM PMA; c, $p < 0.05$ versus 20 nM PMA.

Concentration-Dependent Characteristics of PMA-Induced CRF_1 Receptor Desensitization in Y79 Cells. We next established the concentration-dependence of the desensitizing effect of a 15-min PMA exposure on the ability of CRF to stimulate cAMP accumulation. CRF-stimulated cAMP accumulation was first observed to decrease at 10 nM PMA (80.8 ± 2.0% control; $p < 0.001$) and reached a nadir at 100 nM PMA (48.1 ± 3.6% control; $p < 0.001$) (Fig. 4). The EC_{50} values for the PMA-induced CRF_1 receptor desensitization data were 15 nM. When Y79 cells were coincubated for 15 min with 10 nM CRF (which alone did not significantly desensitize CRF_1 receptors) and 10 nM PMA, the magnitude of CRF_1 receptor desensitization (48.3 ± 2.6%; $p < 0.05$) was significantly greater than that caused by 15-min pretreatment with 10 nM PMA (32.8 ± 3.7%) (Fig. 5). Concentration-dependent CRF_1 receptor desensitization was also observed in Y79 cells exposed to PMA (0–1 μM) for 1 h ($p < 0.0001$; data not shown).

Effect of 1,2-Dioctanoyl-*sn*-glycerol (DOG) on CRF-Stimulated cAMP Accumulation. DOG structurally resembles the diacylglycerols formed when membrane phosphoinositides are hydrolyzed during G_q-coupled GPCR activation, and represents a more "physiological" signal stimulating PKC translocation to the membrane. Y79 cells pretreated with 100 μM DOG for 30 min (12.7 ± 0.4 pmol/10^6 cells; $p < 0.001$) and 1 h (10.0 ± 0.3 pmol/10^6 cells; $p < 0.001$) exhibited time-dependent reductions in the magnitude of CRF-stimulated cAMP accumulation compared with control cells (17.0 ± 0.4 pmol/10^6 cells) (Fig. 6). The magnitude of CRF_1 receptor desensitization resulting from a 1-h DOG exposure (40.4 ± 2.1%; $p < 0.05$) was significantly greater than that observed after a 3-h DOG exposure (29.0 ± 2.7% decrease) (Fig. 6).

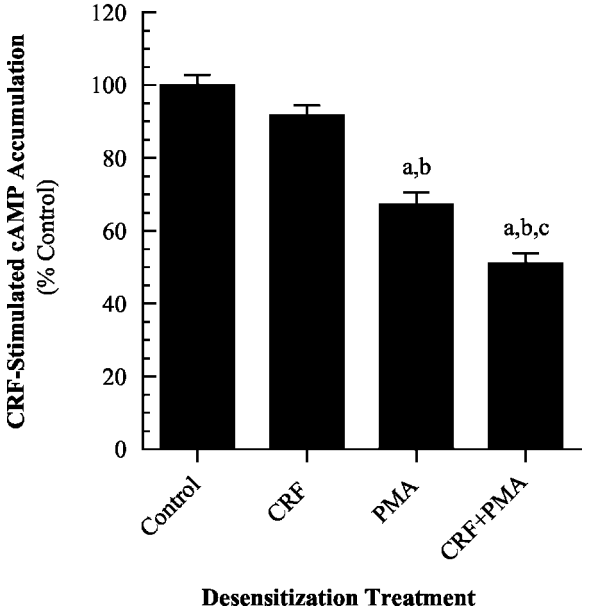

Fig. 5. CRF_1 receptor desensitization magnitudes resulting from a 15-min exposure to 10 nM PMA and/or 10 nM CRF. Data are mean ± S.E.M. of values expressed as percentage of control (n = 10 replicates per treatment group). These data were replicated in two independent experiments. By ANOVA, there were significant differences across the groups (F = 51.99, $p < 0.0001$). The following post hoc differences were found to be statistically significant between groups: a, $p < 0.001$ versus control; b, $p < 0.001$ versus 10 nM CRF 15 min; c, $p < 0.05$ versus 10 nM PMA 15 min.

Expression of Protein Kinase C Isoforms in Y79 Cells. Retinoblastoma cell lysates were immunoprobed with antibodies that selectively recognize specific PKC isoforms to

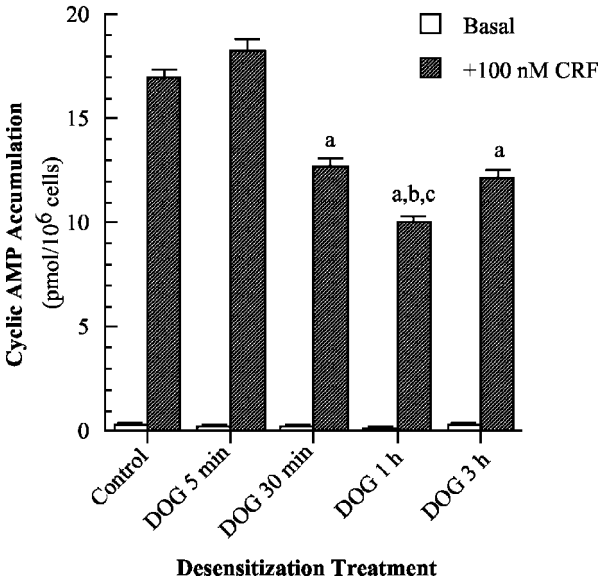

Fig. 6. Time courses for CRF_1 receptor desensitization in Y79 cells exposed to 100 μM 1,2-dioctanoyl-*sn*-glycerol (DOG). Data are mean ± S.E.M. of values expressed pmol/10^6 cells for the stimulation of cAMP accumulation by 100 nM CRF (n = 10 per group) replicated in two independent experiments. By ANOVA, there were significant differences across the groups ($F = 206.8$, $p < 0.0001$). Significant cAMP responses to 100 nM CRF ($p < 0.001$) were observed in control cells and cells pretreated with DOG for 5 min, 30 min, 1 h, and 3 h. The following post hoc differences were also found to be statistically significant between groups: a, $p < 0.001$ versus control; b, $p < 0.01$ versus DOG 30 min; c, $p < 0.05$ versus DOG 3 h.

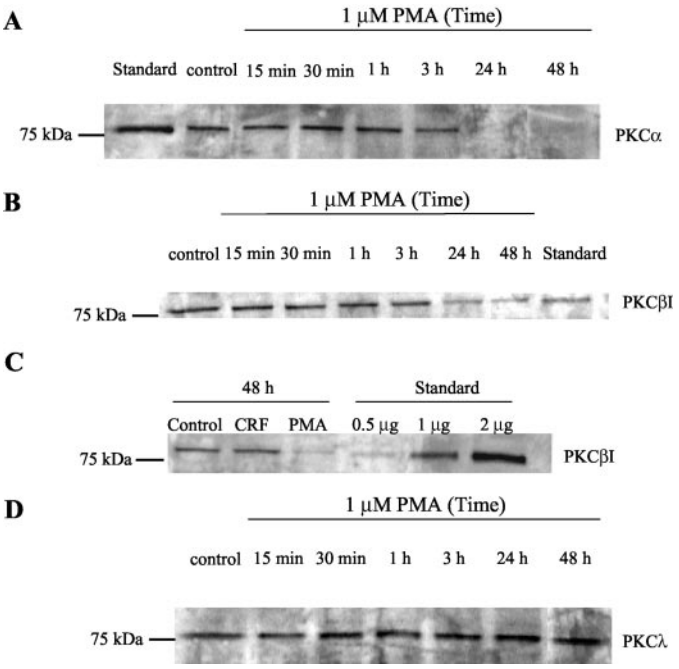

Fig. 7. Down-regulation of α- and βI-isoforms of protein kinase C in Y79 cells during exposure to 1 μM PMA. Representative immunoblots (20–30 μg cell protein per lane) depicting changes in the expression of PKCα (A), PKCβI (B and C), or PKCλ (D) in Y79 cells exposed to 1 μM PMA, 1 μM CRF, or vehicle. Retinoblastoma PKCα, PKCβI, or PKCλ antibody-immunodetected bands comigrated in parallel with rat cerebrum standards at 80 to 82 kDa, consistent with the known molecular weights of these two PKC isoforms. Lysates of rat cerebrum (α-, βI-, and λ-isoforms) were used as internal controls for each immunoblot. These data were replicated in three independent experiments.

determine the pattern of protein kinase C expression in Y79 cells. Well defined retinoblastoma lysate bands migrated to positions consistent with the known molecular weights of PKCα (Fig. 7A), PKCβI (Fig. 7, B and C), and PKCλ (Fig. 7D). Expression of PKCβII, PKCδ, PKCι ((the mouse homolog of PKCλ), and RACK1 (a receptor protein that anchors protein kinase C to the membrane) was also detected in Y79 cells (data not shown). However, no immunoreactive bands were detected with specific antibodies for PKCγ, PKCϵ, PKCη, PKCθ, and DGKθ (data not shown). When additional immunoblotting experiments were performed using a polyclonal phospho-specific antibody (P500) targeting the phosphorylated activation loop of all PKC isoforms (Dutil and Newton, 2000), several bands migrating to 75–80 kDa were detected in Y79 cells (data not shown). Consequently, phosphorylated "mature" PKC species are present in the retinoblastoma cytosol. Only PKC isoforms with a phosphorylated activation loop are capable of catalyzing PKC-mediated phosphorylation of cellular proteins (Dutil and Newton, 2000).

The levels of PKCα and βI proteins were depleted in Y79 cells exposed to PMA for 24 h or 48 h compared with control cells (Fig. 7, A–C). In contrast, incubating Y79 cells with 1 μM CRF for 24 h or 48 h did not decrease the expression of PKCα (data not shown) or PKCβI (Fig. 7C). This duration of CRF treatment produces >90% desensitization of retinoblastoma CRF_1 receptors and a 2- to 3-fold up-regulation of GRK3 expression (Hauger et al., 1997; Dautzenberg et al., 2002).

Effect of Down-Regulating PKC Expression on PMA-Induced CRF_1 Receptor Desensitization. Down-regulation of PKC by chronic phorbol ester treatment is another method for confirming that protein kinase C plays a role in receptor desensitization. Prolonged exposure of cells to PMA induces proteolysis, thereby depleting classical and novel, but not atypical, isoforms of PKC (Tanaka and Nishizuka, 1994). When retinoblastoma cells were pretreated with 1 μM PMA for 1 h, the magnitude of CRF-stimulated cAMP accumulation (11.4 ± 0.3 pmol/10^6 cells; $p < 0.001$) again decreased by 60% compared with the CRF-stimulated cAMP response in control cells (Fig. 8). However, a large decrement in the ability of PMA to desensitize CRF_1 receptors was observed in Y79 cells chronically exposed to 1 μM PMA for 24 h (61.8 ± 4.9% of control) before 1-h PMA pretreatment and subsequent 15-min CRF stimulation (Fig. 8A). The similar magnitudes of CRF-stimulated cAMP accumulation in control Y79 cells (20.1 ± 0.6 pmol/10^6 cells) and cells subjected to the 48-h PMA exposure (22.0 ± 0.5 pmol/10^6 cells) indicated that chronic PMA treatment abolished PMA-induced CRF_1 receptor desensitization (Fig. 8A). Exposure of Y79 cells to PMA for 1 h, 24 h, or 48 h did not alter forskolin-stimulated cAMP accumulation (Fig. 8, A and B). As described above, the levels of PKCα, βI, and βII proteins were depleted in Y79 cells exposed to PMA for 24 h or 48 h compared with control cells, while PKCλ expression did not change (Fig. 7, A–C). Since PKC down-regulation by 48-h PMA treatment abolished PMA-induced CRF_1 receptor desensitization, typical but not atypical PKC isoforms desensitize retinoblastoma CRF_1 receptors without altering adenylyl cyclase activity. Finally, in preliminary experiments, the magnitudes of homologous CRF_1 receptor desensitization did not differ in control Y79 cells and cells in which PKC was down-regulated by 48-h PMA exposure (data not shown).

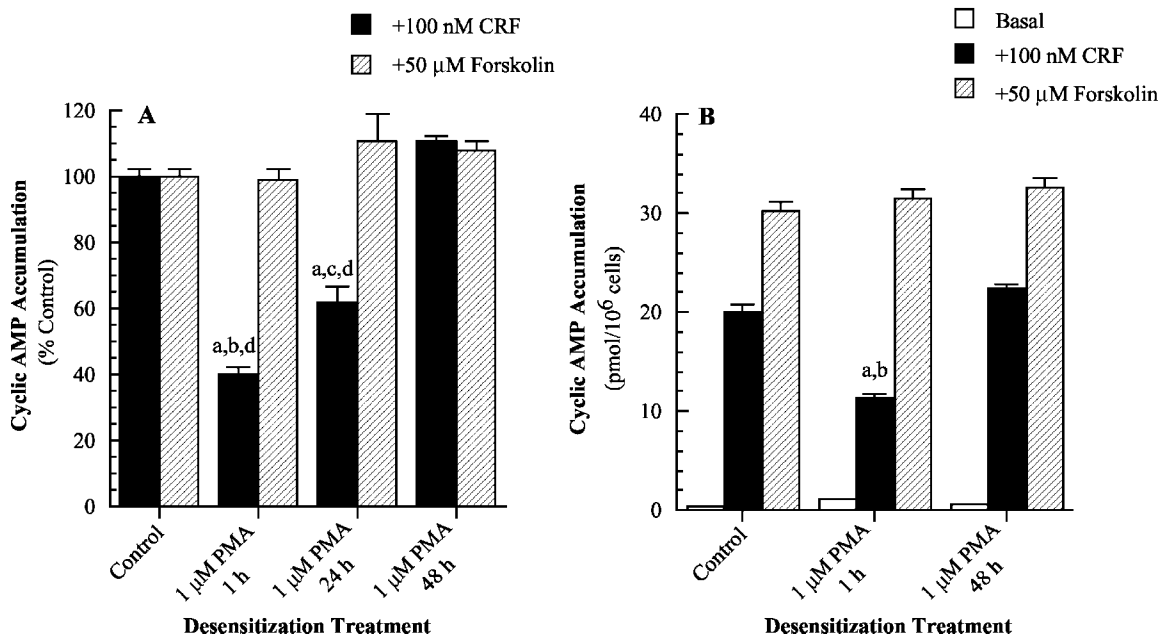

Fig. 8. Effect of down-regulating protein kinase C expression on CRF_1 receptor desensitization in Y79 cells induced by a 1-h exposure to 1 μM PMA. A, data (mean ± S.E.M.) expressed as percentage of control for the stimulation of cAMP accumulation by 100 nM CRF (n = 6–16 per group) were collected in three independent experiments. By ANOVA, there were significant differences across the groups ($F = 52.5$, $p < 0.0001$). The following post hoc differences were found to be statistically significant between groups: a, $p < 0.001$ versus control; b, $p < 0.01$ versus PMA 24 h; c, $p < 0.001$ versus PMA 48 h; d, $p < 0.001$ versus PMA 24 h-forskolin. B, representative experiment for 48-h PMA exposure. Data are mean ± S.E.M. of basal and CRF- and forskolin-stimulated cAMP levels (pmol/10^6 cells) (n = 6 per group). By ANOVA, there were significant differences across the groups ($F = 288.4$, $p < 0.0001$). Significant cAMP responses to 100 nM CRF ($p < 0.001$) and 50 μM forskolin ($p < 0.001$) were observed in control cells and cells pretreated with PMA for 1, 24, and 48 h. In addition, forskolin-stimulated cAMP accumulation was significantly greater than CRF-stimulated cAMP accumulation in control and PMA-treated cells ($p < 0.001$). However, the 48-h PMA exposure abolished PMA-induced CRF_1 receptor desensitization. The following post hoc differences were also found to be statistically significant between groups: a, $p < 0.001$ versus control; b, $p < 0.001$ versus PMA 48 h.

Effect of Protein Kinase C Inhibition on Homologous and Heterologous CRF_1 Receptor Desensitization. The PKC inhibitors BIM (Fig. 9A) and staurosporine (500 nM; data not shown) had no effect on the homologous desensitization of retinoblastoma CRF_1 receptors. A 15-min exposure to 100 nM CRF resulted in similar reductions in the magnitude of cAMP accumulation following subsequent restimulation with 100 nM CRF in Y79 cells pretreated for 30 min with vehicle (30.2 ± 2.3%) or 2 μM BIM (36.9 ± 1.8%) (Fig. 9A). In contrast, pretreating Y79 cells with BIM significantly inhibited PMA-induced CRF_1 receptor desensitization (Fig. 9B). CRF-stimulated cAMP accumulation decreased in Y79 cells

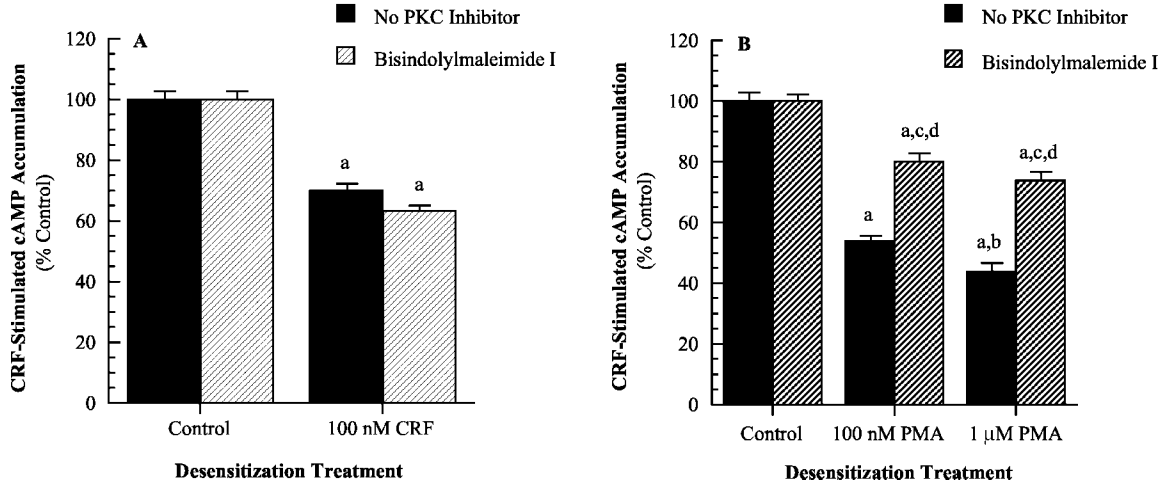

Fig. 9. A, effect of pretreating Y79 cells with the PKC inhibitor bisindolylmaleimide I (2 μM) for 30 min on homologous CRF_1 receptor desensitization induced by 15-min exposure to 100 nM CRF. Data are mean ± S.E.M. of values expressed as percentage of control (n = 19–20 replicates per treatment group) obtained in two separate experiments. By ANOVA, there were significant differences across the groups ($F = 66.7$, $p < 0.0001$). The following post hoc differences were found to be statistically significant between groups: a, $p < 0.001$ versus control. B, effect of pretreating Y79 cells with the PKC inhibitor bisindolylmaleimide I (2 μM) for 30 min on PMA-induced CRF_1 receptors in Y79 cells. Data are mean ± S.E.M. of values expressed as percentage of control (n = 7–17 replicates per treatment group) obtained in two separate experiments. After the 30-min pretreatment with 2 μM BIM was completed, Y79 cells were exposed to 100 nM PMA or 1 μM PMA for 15 min. By ANOVA, there were significant differences across the groups (F = 80.2, $p < 0.0001$). The following post hoc differences were found to be statistically significant between groups: a, $p < 0.001$ versus control; b, $p < 0.05$ versus 100 nM PMA-no PKC inhibitor; c, $p < 0.001$ versus 100 nM PMA-no PKC inhibitor; d, $p < 0.001$ versus 1 μM PMA-no PKC inhibitor.

subjected to a 15-min exposure to 100 nM (53.9 ± 1.5% of control; $p < 0.001$) or 1 μM PMA (44.1 ± 2.6% of control; $p < 0.001$) (Fig. 9B). A 30-min pretreatment with 2 μM BIM inhibited CRF_1 receptor desensitization 56.6 ± 2.2% ($p < 0.001$) and 53.5 ± 2.5% ($p < 0.001$) during a 15-min exposure to 100 nM or 1 μM PMA, respectively (Fig. 9B).

Effect of Protein Kinase C Inhibition on PMA-Induced CRF_1 Receptor Phosphorylation. We have previously reported that a high degree of CRF_1 receptor phosphorylation develops when COS-7 cells transiently transfected with an HA-epitope-tagged CRF_1 receptor cDNA are treated with CRF (Hauger et al., 2000). A 1.82 ± 0.09-fold increase ($p < 0.001$) in the density of the CRF_1 receptor phosphoprotein band (M_r ~70,000) was again observed in transfected COS-7 cells stimulated for 5 min with 1 μM CRF compared with the basal phosphorylation level (data not shown). PMA-induced PKC activation in transfected COS-7 cells strongly phosphorylated the CRF_1 receptor (Fig. 10A). In a total of three experiments, the magnitude of CRF_1 receptor phosphorylation after a 5-min exposure to 200 nM PMA was 2.32 ± 0.21-fold greater compared with basal phosphorylation in control cells not exposed to PMA ($p < 0.001$) (Fig. 10B). A 30-min pretreatment with 2 μM BIM completely abolished PMA-induced CRF_1 receptor phosphorylation (Fig. 10). In addition, the basal level of CRF_1 receptor phosphorylation in untreated control cells was significantly decreased by BIM pretreatment ($p < 0.05$; Fig. 10B).

Discussion

This study establishes that CRF_1 receptors endogenously expressed in human retinoblastoma Y79 cells undergo rapid PKC-mediated desensitization. In a series of experiments, PMA (0–1 μM) desensitized retinoblastoma CRF_1 receptors in a time- and concentration-dependent manner during a 5-min to 1-h pretreatment period. In addition, coincubation of Y79 cells for 15 min with 10 nM PMA (i.e., a concentration producing ~20% desensitization) and 10 nM CRF (which did not result in any significant desensitization) before the 15-min CRF stimulation period desensitized CRF_1 receptors in an additive manner. Since forskolin-stimulated cAMP accumulation did not decrease when Y79 cells were exposed to PMA, PKC-induced CRF_1 receptor desensitization does not appear to involve a direct action of protein kinase C on adenylyl cyclase activity. When full CRF stimulation dose-response curves were generated, the maximum for CRF-stimulated cAMP accumulation decreased by more than 50% in Y79 cells exposed to 1 μM PMA for 1 h. The inability of 4α-phorbol, which cannot activate PKC, to desensitize retinoblastoma CRF_1 receptors during a 1-h pretreatment period confirmed the specificity of PMA-induced CRF_1 receptor desensitization. Pretreatment of Y79 cells for 30 min or 1 h with DOG, a more "physiological" PKC activator, also caused a large degree of CRF_1 receptor desensitization.

Protein kinase C isoforms comprise a family of serine/threonine kinases that phosphorylate many cellular proteins, including GPCRs (Tanaka and Nishizuka, 1994; Penn and Benovic, 1998; Dempsey et al., 2002; Pierce et al., 2002). Retinoblastoma cells were found to express three conventional PKCs (α, βI, βII), RACK1, which selectively binds PKCβII (Dempsey et al., 2002), and one atypical PKC (λ). Diacylglycerols and phorbol esters bind to the N-terminal C1 regulatory domain of PKCα, βI, and βII (but not PKCλ) and down-regulate these conventional PKC proteins when cells are chronically exposed to PKC activators (Tanaka and Nishizuka, 1994). When the duration of PMA or DOG exposure was extended to 3 h in our study, the magnitude of CRF_1 receptor desensitization was significantly less than that occurring with a 1-h pretreatment, presumably due to in-

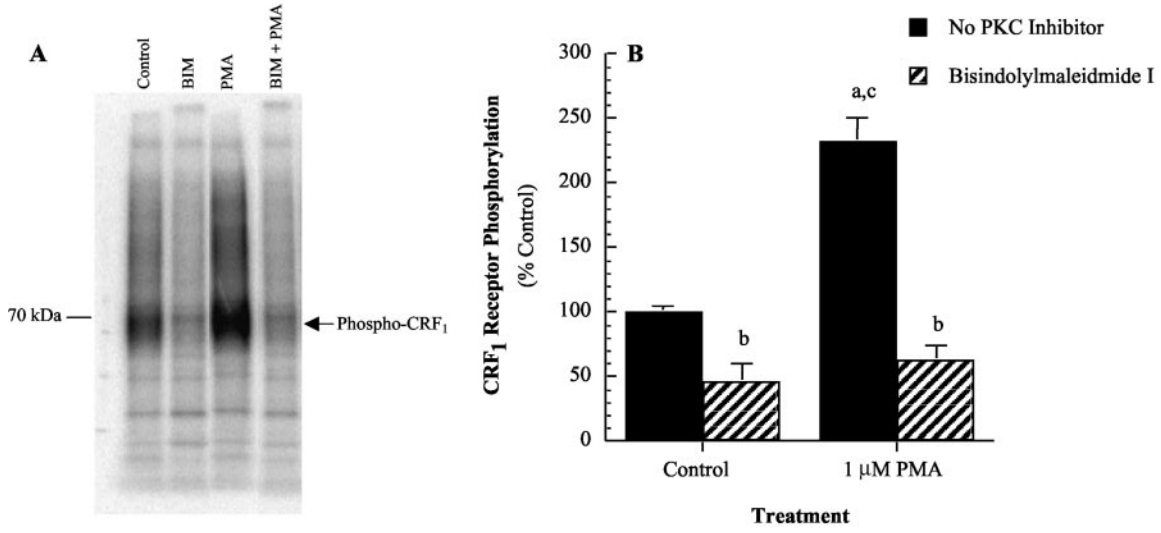

Fig. 10. Effect of the protein kinase C inhibitor bisindolylmaleimide I on PMA-stimulated HA-CRF_1-receptor phosphorylation. A, after a 30-min pretreatment period with vehicle or 2 μM BIM was completed in a representative experiment, ^{32}P-labeled HA-CRF_1R-expressing COS-7 cells were exposed to media (control) or 1 μM PMA for 5 min. Phospho-HA-CRF_1 receptors were immunoprecipitated and resolved by SDS-PAGE. The 60,000 to 70,000 M_r band representing the phosphorylated CRF_1 receptor was 2.5-fold greater in PMA-treated cells (lane 3) compared with the band obtained from cells exposed to vehicle (lane 1). BIM pretreatment abolished PMA-induced HA-CRF_1 receptor phosphorylation (lane 4). B, data (mean ± S.E.M.) from three separate experiments are presented as percentage of increase above basal HA-CRF_1 receptor phosphorylation in control cells (transfected with HA-CRF_1 receptor cDNA but not exposed to PMA). Pretreatment of transfected cells with 2 μM BIM for 30 min lowered basal receptor phosphorylation and blocked the ability of PMA to stimulate receptor phosphorylation. By ANOVA, there were significant differences across the groups ($F = 35.5$, $p < 0.0001$). The following post hoc differences were found to be statistically significant between groups: a, $p < 0.001$ versus PMA-no PKC inhibitor; b, $p < 0.05$ versus control-no PKC inhibitor; c, $p < 0.001$ versus PMA-BIM.

creased proteolytic degradation of PKC proteins. Subsequent experiments confirmed this possibility by demonstrating that PMA-induced CRF_1 receptor desensitization was abolished in Y79 cells where PKCα and PKCβI protein levels were depleted by a 48-h PMA exposure. PKCλ protein levels were not reduced in Y79 cells chronically exposed to PMA. Chronic PMA treatment can also down-regulate expression of the novel isoform PKCδ (Tanaka and Nishizuka, 1994), which is expressed in Y79 cells. Therefore, our data indicate that α-, β-, and possibly δ-isoforms of PKC desensitize retinoblastoma CRF_1 receptors.

BIM acts as a competitive inhibitor at the ATP binding site within the catalytic domains of many PKC isoforms including PKCα, PKCβ, PKCδ, PKCϵ, and PKCζ (Tanaka and Nishizuka, 1994). Although 2 μM BIM pretreatment did not alter homologous CRF_1 receptor desensitization, it markedly inhibited PMA-induced CRF_1 receptor desensitization. This suggests that a PKC-dependent mechanism contributes to heterologous, but not homologous, desensitization of CRF_1 receptors.

CRF_1 receptors in brain and transfected mouse fibroblast cells can couple to both G_s and G_q proteins, thereby activating both cAMP and phospholipase C pathways (Dieterich et al., 1996; Hillhouse et al., 2002). In Leydig cells and placenta, CRF_1 receptors may signal via G_q-mediated stimulation of phospholipase C and formation of IPs without generating an intracellular cAMP-dependent signal (Ulisse et al., 1990; Karteris et al., 2000). Taken together, these findings suggest that the CRF_1 receptor can couple to G_q and signal via the protein kinase C cascade in certain cell types (Kiang et al., 1994; Dautzenberg and Hauger, 2002). However, inositol phosphate generation was not increased in Y79 cells stimulated with CRF and inhibition of PKC in Y79 cells by BIM pretreatment failed to block homologous CRF_1 receptor desensitization. Thus, it seems likely that the CRF_1 receptor expressed in retinoblastoma cells couples to G_s but not to G_q. Similar findings have been observed for the pituitary adenylate cyclase-activating polypeptide type 1 receptor (PAC_1). The PAC_1 receptor exclusively couples to G_s in Y79 cells (Olianas et al., 1996; Dautzenberg et al., 1999), while the PAC_1 receptor couples to G_s and G_q in other cells (Harmar et al., 1998).

We determined whether an HA-epitope-tagged CRF_1 receptor recombinantly expressed in COS-7 cells could be phosphorylated by protein kinase C to establish a possible mechanism for PMA-induced CRF_1 receptor desensitization. A high degree of CRF_1 receptor phosphorylation was detected in transfected COS-7 cells during a 5-min exposure to PMA—an effect that was completely abolished by 30-min pretreatment with BIM. We previously observed that CRF exposure, but not forskolin-induced PKA activation or ionomycin-induced stimulation of Ca^{2+}/calmodulin-dependent kinases, causes phosphorylation of the CRF_1 receptor in COS-7 cells, presumably due to a G protein receptor kinase mechanism (Hauger et al., 2000). Similarly, stimulation of PKA activity by forskolin or dibutyryl cAMP failed to desensitize CRF_1 receptors in Y79 and IMR-32 cells (Dautzenberg et al., 2001a; Roseboom and Kalin, 2001).

Recently, mice with a targeted deletion of the PKCβ gene were found to exhibit a deficit in fear conditioning (Weeber et al., 2000). In BALB/c mice, context-dependent fear conditioning was augmented by injecting CRF or activating PKC in the hippocampus, while pretreatment with a PKC inhibitor impaired acute stress-induced enhancement of context-dependent fear conditioning (Blank et al., 2002, 2003). PKC inhibitors also block CRF-induced increases in firing rates of hippocampal neurons in BALB/c mice (Blank et al., 2003). Consequently, PKC-mediated CRF_1 receptor signaling may contribute to the expression of contextual fear. We have previously hypothesized that deficient GRK-mediated CRF_1 receptor desensitization may trigger long-term psychopathology by increasing the sensitivity of and/or prolonging agonist-stimulated CRF_1 receptor signaling in brain pathways mediating fear and anxiety (Dautzenberg and Hauger, 2002). The present data suggest that abnormalities in PKC-mediated phosphorylation and desensitization of CRF_1 receptors may also result in CRF_1 receptor supersensitivity and sensitization of brain CRF neurotransmission, thereby reducing the activation threshold and increasing the magnitude and duration of fear and anxiety responses.

In conclusion, we have demonstrated that rapid desensitization of retinoblastoma CRF_1 receptors occurred when protein kinase C is activated. The presence of two potential PKC phosphorylation sites (Ser^{386} and Ser^{408}) in the C-terminal cytoplasmic tail of the CRF_1 receptor suggests that the phosphorylation of the CRF_1 receptor by protein kinase C observed in this study may mediate this desensitization process (Chen et al., 1993; Dautzenberg et al., 2001b). However, depending on the cellular background, PKC-mediated CRF_1 receptor desensitization may occur via heterologous cross-talk with one or more G_q-coupled GPCRs or via a homologous mechanism whereby the agonist-activated CRF_1 receptor couples to the G_q protein and subsequently activates its phosphorylation by PKC. In a recent study it was found that activation of oxytocin receptors causes heterologous desensitization of CRF-stimulated cAMP accumulation in human myometrial cells via a PKC-dependent mechanism (Grammatopoulos and Hillhouse, 1999). Based on previous reports of cross-talk between receptor signaling pathways (Selbie and Hill, 1998; Budd et al., 1999; Thakker and Standifer, 2002), activation of heterologous GPCRs may result in cross-phosphorylation, desensitization, and possibly internalization of CRF_1 receptors, thereby shifting the stress response to other receptor mechanisms. Future studies will be directed at the identification of GPCRs that desensitize retinoblastoma CRF_1 receptors via PKC-mediated cross-talk.

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References

Abou-Samra A-B, Harwood JP, Manganiello VC, Catt KJ, and Aguilera G (1987) Phorbol 12-myristate 13-acetate and vasopressin potentiate the effect of corticotropin-releasing factor on cyclic AMP production in rat anterior pituitary cells. *J Biol Chem* **262:**1129–1136.

Bhattacharyya S, Puri S, Miledi R, and Panicker MM (2002) Internalization and recycling of 5-HT2A receptors activated by serotonin and protein kinase C-mediated mechanisms. *Proc Natl Acad Sci USA* **99:**14470–14475.

Blank T, Nijholt I, Eckart K, and Spiess J (2002) Priming of long-term potentiation in mouse hippocampus by corticotropin-releasing factor and acute stress: implications for hippocampus-dependent learning. *J Neurosci* **22:**3788–3794.

Blank T. Nijholt I, Grammatopoulos DK, Randeva HS, Hillhouse HS, and Spiess J (2003) Modulation of neuronal excitability and associative learning by different

corticotropin releasing factor-signaling cascades in mouse hippocampus. *J Neurosci* **23:**700–707.
Budd DC, Challiss RAJ, Young KW, and Tobin AB (1999) Cross talk between m3-muscarinic and β2-adrenergic receptors at the level of receptor phosphorylation and desensitization. *Mol Pharmacol* **56:**813–823.
Chen R, Lewis KA, Perrin MH, and Vale WW (1993) Expression cloning of a human corticotropin-releasing-factor receptor. *Proc Natl Acad Sci USA* **90:**8967–8971.
Cliang A, Laporte SA, Caron MG, and Lefkowitz RJ (2002) Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and β-arrestin proteins. *Prog Neurobiol* **66:**61–79.
Dautzenberg FM, Braun S, and Hauger RL (2001a) GRK3 mediates homologous desensitization of CRF1 receptors: a potential mechanism regulating stress adaptation. *Am J Physiol Regul Integr Comp Physiol* **280:**R935–R946.
Dautzenberg FM and Hauger RL (2001) G protein receptor kinase 3 and protein kinase C mediate the homologous and heterologous desensitization, respectively, of the PACAP receptor, type 1 in human Y-79 retinoblastoma cells: absence of protein kinase A action in PAC_1 receptor desensitization. *Neuropharmacology* **40:**394–407.
Dautzenberg FM and Hauger RL (2002) The CRF peptide family and their receptors: yet more partners discovered. *Trends Pharmacol Sci* **23:**71–77.
Dautzenberg FM, Kilpatrick GJ, Hauger RL, and Moreau J-L (2001b) Molecular biology of the CRH receptors—in the mood. *Peptides* **22:**753–760.
Dautzenberg FM, Mevenkamp G, Wille S, and Hauger RL (1999) N-terminal splice variants of the type I PACAP receptor: isolation, characterization and ligand binding/selectivity determinants. *J Neuroendocrinol* **11:**941–949.
Dautzenberg FM, Wille S, Braun S, and Hauger RL (2002) GRK3 regulation during CRF- and urocortin-induced CRF_1 receptor desensitization. *Biochem Biophys Res Commun* **298:**303–308.
Dempsey EC, Newton AC, Mochly-Rosen D, Fields AP, Reyland ME, Insel PA, and Messing RO (2002) Protein kinase C isozymes and the regulation of diverse cell responses. *Am J Physiol Lung Cell Mol Physiol* **279:**L429–L438.
Dieterich KD, Grigoriadis DE, and DeSouza EB (1996) Homologous desensitization of human corticotropin-releasing factor1 receptor in stable transfected mouse fibroblast cells. *Brain Res* **710:**287–292.
Dutil EM and Newton AC (2000) Dual role of pseudosubstrate in the coordinated regulation of protein kinase C by phosphorylation and diacylglycerol. *J Biol Chem* **275:**10697–10701.
Grammatopoulos D and Hillhouse EW (1999) Activation of protein kinase C by oxytocin inhibits the biological activity of the human myometrial corticotropin-releasing hormone receptor at term. *Endocrinology* **140:**585–594.
Grigoriadis D, Haddach M, Ling N, and Saunders J (2001) The CRF receptor: structure, function and potential for therapeutic intervention. *Curr Med Chem–CNS Agents* **1:**63–97.
Harmar AJ, Arimura A, Gozes I, Journot L, Laburthe M, Pisegna JR, Rawlings SR, Robberecht P, Said SI, Sreedharan SP, et al. (1998) International union of pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptides. *Pharmacol Rev* **50:**265–270.
Hauger RL, Dautzenberg FM, Flaccus A, Liepold T, and Spiess J (1997) Regulation of corticotropin-releasing factor receptor function in human Y-79 retinoblastoma cells: rapid and reversible homologous desensitization but prolonged recovery. *J Neurochem* **68:**2308–2316.
Hauger RL, Smith RD, Braun S, Dautzenberg FM, and Catt KJ (2000) Rapid agonist-induced phosphorylation of the human CRF receptor, type 1: a potential mechanism for homologous desensitization. *Biochem Biophys Res Commun* **268:**572–576.
Karteris E, Grammatopoulos D, Randeva H, and Hillhouse EW (2000) Signal transduction characteristics of the corticotropic-releasing hormone receptors in the feto-placental unit. *J Clin Endocrinol Metab* **85:**1989–1996.
Kiang JG, Wang X, and McClain (1994) Corticotropin-releasing factor increases protein kinase C activity by elevating membrane-bound α and β isoforms. *Chin J Physiol* **37:**105–110.
Kohout TA and Lefkowitz RJ (2003) Regulation of G protein-coupled receptor kinases and arrestins during receptor desensitization. *Mol Pharmacol* **63:**9–18.
Krupnick JG and Benovic JL (1998) The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annu Rev Pharmacol Toxicol* **38:**289–319.
Mandyam CD, Thakker DR, Christensen JL, and Standifer KM (2002) Orphanin FQ/nociceptin-mediated desensitization of opioid receptor-like 1 receptor and m opioid receptors involves protein kinase C: a molecular mechanism for heterologous cross-talk. *J Pharmacol Exp Ther* **302:**502–509.
Menard L, Ferguson SSG, Lefkowitz RJ, Caron MG, and Barak LS (1997) Synergistic regulation of β_2-AR receptor sequestration: intracellular complement of βARK and β-arrestin determine kinetics of internalization. *Mol Pharmacol* **51:**800–808.
Olianas MC, Ennas MG, Lampis G, and Onali P (1996) Presence of pituitary adenylate cyclase-activating polypeptide receptors in Y-79 retinoblastoma cells. *J Neurochem* **67:**1293–1300.
Olivares-Reyes JA, Smith RD, Hunyady L, Shah BH, and Catt KJ (2001) Agonist-induced signaling, desensitization and internalization of a phosphorylation-deficient AT_{1A} angiotensin receptor. *J Biol Chem* **276:**37761–37768.
Oppermann M, Mack M, Proudfoot AEI, and Olbrich H (1999) Differential effects of CC chemokines on CC chemokine receptor 5 (CCR5) phosphorylation and identification of phosphorylation sites on the CCR5 carboxyl terminus. *J Biol Chem* **274:**8875–8885.
Penn RB and Benovic JL (1998) Mechanisms of receptor regulation, in *Handbook of Physiology*, *Section 7: Endocrinology* (Conn PM ed) vol I, pp 125–164, Oxford University Press, New York.
Perrin MH and Vale W (2002) Corticotropin-releasing factor receptors, in *Understanding G Protein-Coupled Receptors and Their Role in the CNS* (Pangalos MN and Davies CH eds) pp 505–526, Oxford University Press, New York.
Pierce KL, Premont RT, and Lefkowitz RJ (2002) Seven-transmembrane receptors. *Nature (Lond) Rev* **3:**639–650.
Roseboom PH and Kalin N (2001) Persistent corticotropin-releasing factor(1) receptor desensitization and downregulation in the human neuroblastoma cell line IMR-32. *Brain Res Mol Brain Res* **92:**115–127.
Selbie LA and Hill SJ (1998) G protein-coupled-receptor cross-talk: the fine-tuning of multiple receptor-signaling pathways. *Trends Pharmacol Sci* **19:**87–93.
Tanaka C and Nishizuka Y (1994) The protein kinase C family for neuronal signaling. *Annu Rev Neurosci* **17:**551–567.
Thakker DR and Standifer KM (2002) Induction of G protein-coupled receptor kinases 2 and 3 contributes to the cross-talk between m and ORL1 receptors following prolonged agonist exposure. *Neuropharmacology* **43:**979–990.
Ulisse S, Fabbri A, Tinajero JC, and Dufau ML (1990) A novel mechanism of action of corticotropin releasing factor in rat Leydig cells. *J Biol Chem* **265:**1964–1971.
Weeber EJ, Atkins CM, Selcher JC, Varga AW, Mirnikjoo B, Paylor R, Leitges M, and Sweatt JD (2000) A role for the β isoform of protein kinase C in fear conditioning. *J Neurosci* **20:**5906–5914.

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